

Original Article

Effects of *In vitro* hemodilution, hypothermia and rFVIIa addition on coagulation in human blood

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Abstract: Introduction: Coagulopathy can occur after hemorrhage, trauma and resuscitation, and has been associated with dilution of coagulation factors and hypothermia. Recombinant activated Factor VII (rFVIIa) has been used, often as a last resort, to improve hemostasis in trauma/hemorrhage patients with coagulopathy. The aim of this study was to further characterize the effects of rFVIIa on various coagulation parameters and the influence of temperature and hemodilution. Methods: Whole blood from healthy human volunteers was incubated in a combination of three conditions: undiluted or diluted 40% with either lactated Ringer's solution or Hextend, at 37°C or 34°C, and with and without rFVIIa (1.26 µg/ml, final concentration). Blood or plasma, as appropriate, was measured for coagulation by thrombin generation, thromboelastography (TEG), prothrombin Time (PT) and activated partial thromboplastin (aPTT). Results: Incubation of plasma at 34°C significantly elevated thrombin generation, and prolonged PT and aPTT. Dilution of blood or plasma with 40% Hextend, but not lactated Ringer's, had a significant effect on TEG parameters, and prolonged PT and aPTT. In control conditions (37°C, 0 dilution), the addition of rFVIIa to human plasma or whole blood led to a significant change in all TEG parameters, and Lagtime for thrombin generation, but not to PT or aPTT. Conclusion: These data show that thrombin generation is affected by hypothermia, but not 40% dilution. TEG is affected by 40% dilution with Hextend, but not by hypothermia. PT and aPTT are significantly affected by both hypothermia and dilution. Recombinant FVIIa caused a greater change in thrombin generation at 34°C as compared to 37°C, and a greater change in PT at 40% dilution, suggesting that the effect of rFVIIa on coagulation is both temperature and dilution dependant.

Keywords: TEG, thrombin generation, PT, aPTT

Introduction

Coagulopathy is a clinical condition defined as a disorder of hemostasis. It can be manifest as a hypo- or hyper-coagulation state. Coagulopathy has been well documented in hemorrhage/trauma patients as a consequence of acidosis, hypothermia, dilution from resuscitation. In addition, a trauma-induced coagulopathy has been observed and is associated with a poor predictable outcome in trauma patients [1-4]. Restoration and maintenance of circulating blood volume, as well as correction of the acidosis, hypothermia and impaired coagulation are critical steps in the successful resuscitation of severely injured patients [5-10].

Fluid resuscitation has been a long standing therapy for trauma and hemorrhagic shock to

restore blood volume, cardiac output and flow to the microcirculation [11-16]. Lactated Ringer's (LR) and Hextend (HX) are the primary fluids used by many trauma units and the US Army for pre-hospital resuscitation [17]. HX, a hetastarch-based product in a balanced electrolyte solution with glucose, was selected for the battlefield resuscitation of casualties in shock by the Tactical Combat Casualty Care committee, for its volume sparing effects as compared to LR. In patients with severe hemorrhage, large volume infusions of crystalloids or colloids have led to significant hemodilution and coagulopathy [4, 18, 19]. This can be experimentally induced in animals [14, 20] and allows for the study of this pathophysiology.

Over the past several years, rFVIIa has received much attention as a novel hemostatic agent for

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treating coagulopathy associated with hemorrhage and trauma [15, 17-24]. Infusion of rFVIIa results in an enhancement of thrombin generation on the platelet surface at the site of injury independent of the presence of Factor VIII/IX [21, 25, 26]. Thrombin generation reflects the action of the initial hemostatic mechanism and is a reliable test that could be used experimentally to determine the effects of fluid resuscitation and temperature on coagulation.

The aims of this study were to measure thrombin generation and other measures of coagulation, in human blood and plasma *in vitro* 1) with and without rFVIIa, 2) under normal or 40% hemodilution conditions (with either HX or LR), and 3) under normal- or hypothermic temperature conditions (37°C-34°C). Acidosis was not addressed in this study.

Materials and methods

Blood Collection

This study was conducted under a protocol reviewed and approved by the Brooke Army Medical Center Institutional Review Board. Subjects were screened for any known coagulation disorders, any drug prescription or over-the-counter medication or supplement that could have an effect on coagulation function, and pregnancy. Any subject meeting any of these exclusion conditions was not used for this study. After informed consent was acquired, blood specimens were collected from 9 normal healthy volunteers via venipuncture into sodium citrate vacutainers according to standard clinical operating procedures. Blood samples were split and half were centrifugation at 2000g for 10 minutes, plasma separated (platelet poor) and kept at -80°C until testing for thrombin generation, prothrombin time (PT) and activated partial thromboplastin time (aPTT).

PT and aPTT were measured in duplicate by standard clinical methods (BCS Coagulation Analyzer, Dade Behring, Deerfield, IL).

Thrombin generation assay

HX or LR was added to plasma to achieve 40% hemodilution, then incubated at 34°C or 37°C for 15 min in a water bath. Activated recombinant FVII was added to the plasma (1.26 µg/ml, approximately equivalent to a 90 µg/kg clinical

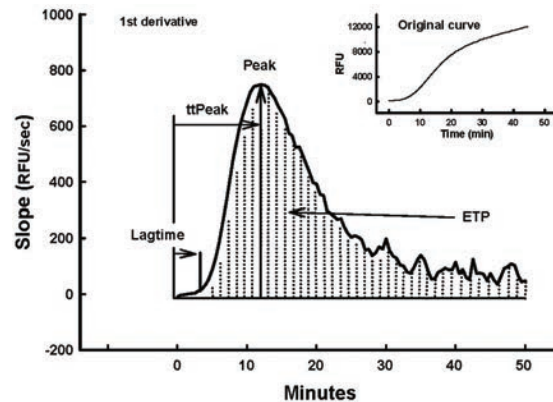


Figure 1. The thrombin generation curve was derived as the 1st derivative of the appearance of the fluorescent product over time (inset). Lagtime, ttPeak, Peak and ETP are illustrated.

dose [14]. Thrombin generation was studied according to the assay of Hemker et al. [25, 27, 28]. Briefly, thrombin generation was triggered by adding a solution containing Tissue Factor (TF), phosphatidyl choline/phosphatidyl serine (PCPS; Hemotologic Technologies), CaCl₂ and Fluorogenic thrombin substrate Z-Gly-Gly-Arg-AMC HCl (Bachem) to 80 µL platelet poor plasma. Samples were run in at least duplicate. The increase in fluorescence intensity, which is proportional to the concentration of the generated thrombin, was monitored every 30 sec for 120 min at 34°C or 37°C using a Microplate Fluorescence Reader SPECTRA max M2 (Molecular Devices, Sunnyvale, CA) with an excitation/emission wavelength of 360nm/460nm. A curve was generated that reflected the conversion of fluorogenic substrate to fluorescent product. The fluorescence data were not corrected for the inner filter effect or substrate consumption. The 1st derivative was calculated (**Figure 1**) to produce the thrombin generation curve. Thrombin generation was expressed using four parameters that described the curve; Lagtime (the initial reaction time when the thrombogram curve reaches 20 RFU/min), Peak (the peak or maximum thrombin generated), ttPeak (the time to reach the peak height of thrombin generated) and endogenous thrombin potential (ETP) or area under the curve (integrated thrombin generation between time 0 -50min).

Thromboelastography (TEG)

All samples were run in triplicate on a Haemo-

Table 1. Effect of temperature and dilution on the change (%change) of various coagulation parameters to rFVIIa

	0 dilution		40% LR		40% Hex	
	37 °C	34 °C	37 °C	34 °C	37 °C	34 °C
Thrombin Generation						
Lagtime (minutes)	-1.8±0.1 (-53±10)	-2.4±0.6 (-82±6)	-2.6±0.4 (-65±7)	-1.9±0.4 (-78±7)	-2.5±0.4 (-75±8)	-2.0±0.3 (-84±5)
ttPeak (minutes)	-2.9±0.7 (-20±4)	-6.1±1.2 (-42±6)	-3.9±0.7 (-25±4)	-3.8±0.6 (-33±3)	-3.9±0.8 (-28±2)	-4.6±0.5 (-40±3)
Peak (RFU/min)	150±31 (21±5)	579±74* (85±20)*	49.5±22.2 (10±4)	288±66* (26±7)	105.5±32.7 (15±6)	360±46* (35±7)
ETP	1297±388 (10±3)	3777±496* (25±5)*	181±278 (2±2)	1196±496 (5±2)	1315±385 (9±3)	2207±173 (11±1)
TEG						
R (minutes)	-3.6±0.9 (-69±11)	-5.3±1.9 (-88±23)	-2.0±0.5 (-45±6)	-3.6±1.0 (-59±9)	-2.9±1.0 (-51±12)	-4.9±1.9 (-70±21)
K (minutes)	-1.2±0.1 (-69±5)	-1.1±0.7 (-64±24)	-0.2±0.3 (-15±11)	-0.6±0.3 (-25±11)	-2.2±0.7 (-84±36)	-2.0±1.3 (-55±35)
α angle (degrees)	12.3±1.0 (19±2)	12.7±3.7 (20±7)	3.6±2.5 (5±5)	6.1±0.6 (11±4)	11.3±3.6 (22±6)	7.7±3.6 (15±8)
MA (mm)	6.8±1.2 (11±2)	4.5±2.1 (7±4)	2.2±1.1 (4±2)	1.9±0.6 (4±1)	7.8±2.5 (16±4)	2.2±1.9 (4±4)
PT (seconds)	-0.2±0.3 (-2±3)	-1.4±0.5 (-12±4)	-2.3±1.1# (-15±10)	-3.7±0.3 (-27±1)	-2.5±0.4# (-20±2)	-2.5±0.3 (-20±2)
aPTT (seconds)	-1.7±0.8 (-6±3)	-4.1±1.3 (-12±4)	-2.9±1.5 (-9±5)	-4.8±0.5 (-14±1)	-4.1±0.4 (-13±1)	-3.8±0.3 (-12±1)

Values represent Mean±SEM of the change to the addition of rFVIIa; *=P<0.05 as compared to 37 °C at that dilution; #=P<0.05 as compared to 37 °C, 0 dilution.

scope Model 5000, (Skokie, IL) at 34 °C and 37 °C, with and without rFVIIa (1.29 µg/ml Novo Nordisk, Denmark, equivalent to 90 µg/kg in vivo dose). The machine was calibrated daily. Before the blood was added, 10 µl of tissue factor (Innovin, diluted 1:500), 20 µl of 0.2 M CaCl₂, and 4.3 µl of 19.2 µg/ml Corn Trypsin Inhibitor (CTI) were added to each cup and allowed to equilibrate. Citrated blood (340 µl) was then added to each cup and the TEGs were started immediately. The tests were terminated 30 minutes after maximum amplitude (MA) was reached.

The parameters that were measured included R, (min, the time it takes for the initial detection of fibrin formation), K (min, the time for formation of the clot); α angle (degree, the kinetics of clot development), and MA (mm, the maximum amplitude (strength) of the developed clot).

Data analysis

Data was analyzed by 2-way and 3-Way ANOVA (rFVIIa, Temperature and dilution were the vari-

ables) followed by multiple comparison analysis using the Tukey's or Sidac-Holms Methods. Statistics were performed by SAS or Sigma-stat®. P < 0.05 was considered significant. Change was calculated as the difference between rFVIIa and control (no rFVIIa), and % change was calculated as the change/control x100% for **Table 1**. The data are expressed as the mean ± standard error of the mean.

Results

Several coagulation parameters were measured in plasma or whole blood with and without rFVIIa, at 37 and 34 °C, and diluted with 0 or 40% Hextend or lactated Ringer's.

Thrombin generation

In undiluted blood, thrombin generation was not significantly affected by temperature (**Figures 2 and 3**). Furthermore, thrombin generation was not affected by dilution with Hextend or LR at 37 °C. However, thrombin generation Peak and ETP were significantly elevated at 34 °C as com-

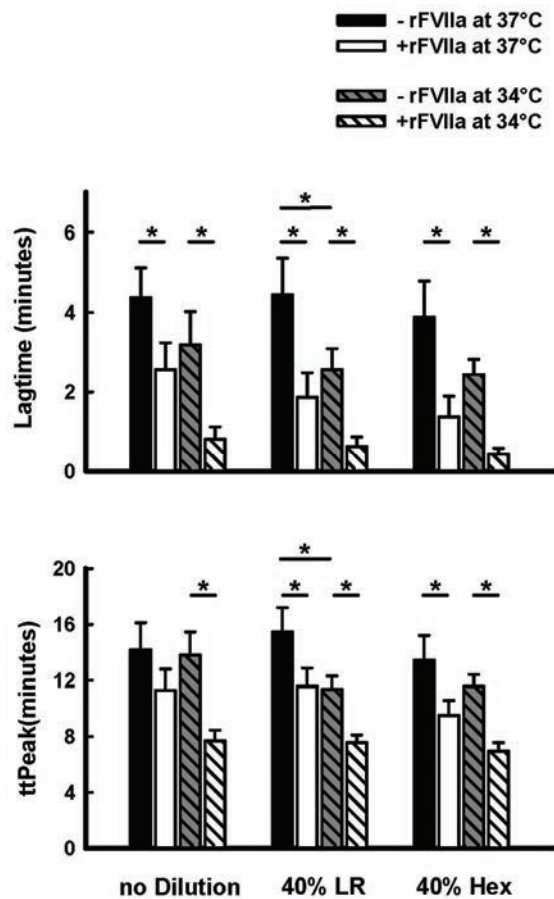


Figure 2. Effect of rFVIIa (1.26 μ g/ml, final concentration in PPP), temperature (34°C and 37°C), and 40% blood hemodilution (Hextend [HX] or lactated ringers [LR]) on Lagtime and ttPeak in human plasma in vitro. Values represent Mean \pm SEM. * $=P<0.05$ between or among groups spanned by the horizontal bars.

pared to 37°C in both 40% diluted groups, whereas lagtime and time to peak were significantly shortened in 34°C plasma diluted with LR as compared to 37°C diluted plasma (Figures 2 and 3). Incubation of plasma with rFVIIa under control conditions (37°C, 0 dilution), significantly reduced thrombin generation Lagtime, but had no significant effect on ttPeak, Peak or ETP (Figures 2 and 3). However, in undiluted plasma at 34°C, rFVIIa significantly shortened lagtime and ttPeak and elevated Peak and ETP (Figures 2 and 3). Similar results were seen in both 40% dilution groups at either temperature (Figures 2 and 3). Generally speaking, temperature had a greater effect on thrombin generation than dilution.

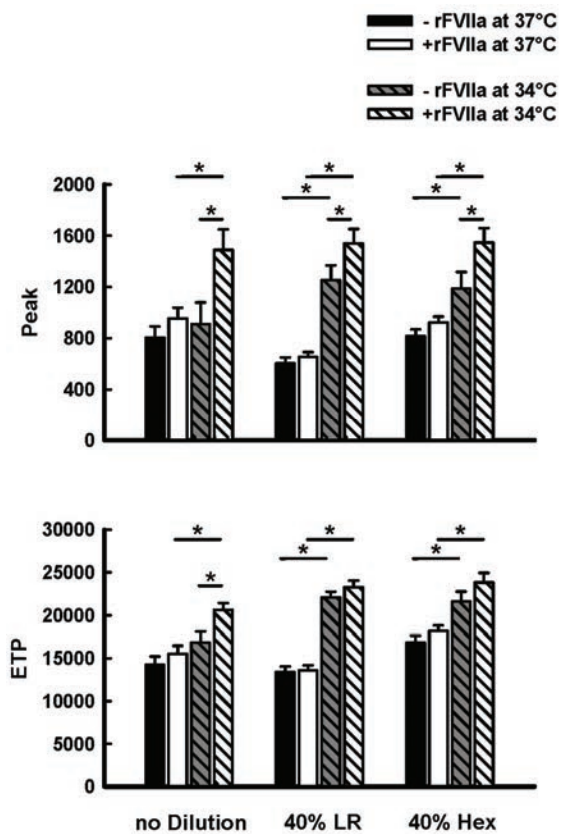


Figure 3. Effect of rFVIIa (1.26 μ g/ml), temperature (34°C and 37°C), and 40% blood hemodilution (Hextend [HX] or lactated ringers [LR]) on Peak and ETP on human plasma in vitro. Values represent Mean \pm SEM. * $=P<0.05$.

Thromboelastography

In undiluted blood, there were no significant changes in any TEG parameter at 34°C compared to 37°C. These non-significant responses to temperature were also observed in both 40% dilution groups (Figures 4 and 5). With respect to 40% dilution, only the Hextend group showed significant prolongation of K time and reduced α angle and MA in comparison to undiluted blood (Figures 4 and 5).

Incubation of undiluted blood with rFVIIa at 37°C significantly shortened R and K times, and increased α angle and MA (Figures 4 and 5). In undiluted blood at 34°C, addition of rFVIIa was still able to significantly reduce R-time and increase α angle. rFVIIa had no significant effect on any TEG parameter in blood diluted 40% with

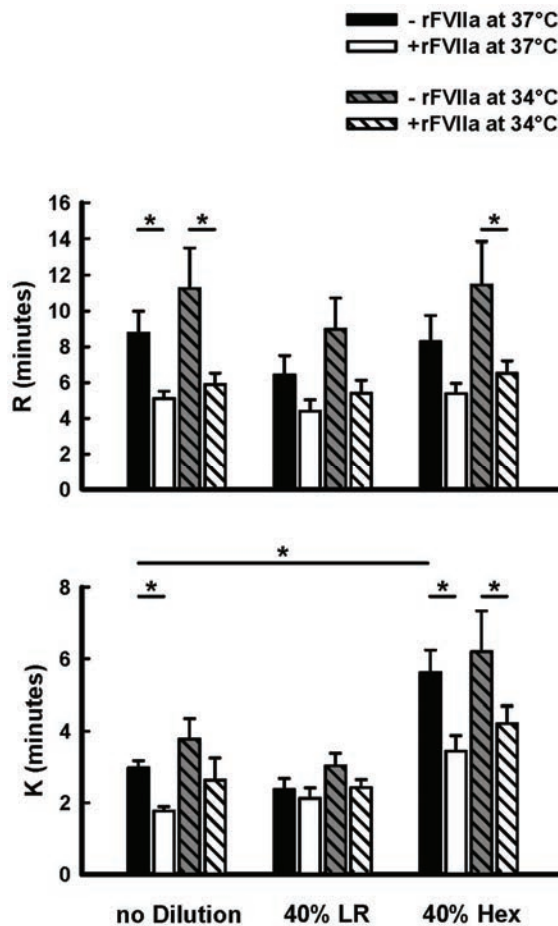


Figure 4. Effect of rFVIIa (1.26µg/ml), temperature (34°C and 37°C), and 40% blood hemodilution (Hextend [HX] or lactated ringers [LR]) on R and K in human blood in vitro. Values represent Mean±SEM. *=P<0.05.

LR at either temperature, whereas the response in blood diluted 40% with Hextend was different (Figures 4 and 5). In this group rFVIIa shortened K-time and increased α angle and MA at 37°C, but only shortened R and K times, with no significant effects on α angle and MA at 34°C.

PT and aPTT

In undiluted blood, PT and aPTT were significantly prolonged in response to the colder temperature, and as expected were prolonged in response to 40% dilution with either fluid in comparison to undiluted blood (Figure 6).

Incubation of undiluted plasma with rFVIIa at 37°C had no effect on PT or aPTT, but did sig-

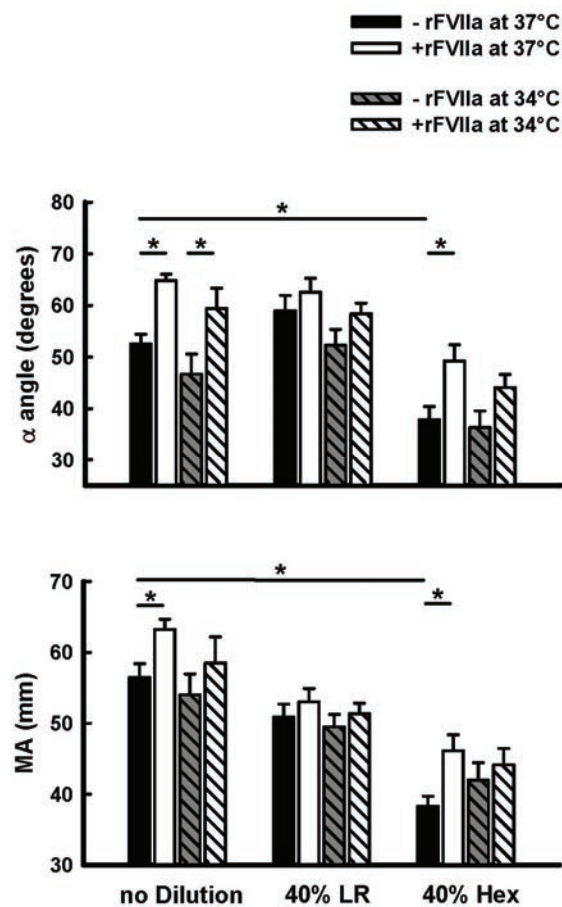


Figure 5. Effect of rFVIIa (1.26µg/ml), temperature (34°C and 37°C), and 40% blood hemodilution (Hextend [HX] or lactated ringers [LR]) on α angle and MA in human blood in vitro. Values represent Mean±SEM. *=P<0.05.

nificantly decrease PT and aPTT after incubation at 34°C (Figure 6). In plasma diluted 40% with either LR or Hextend and at either 37°C or 34°C, addition of rFVIIa also significantly reduced PT and aPTT compared to the respective plasma samples without rFVIIa (Figure 6).

Discussion

The purpose of this study was to evaluate the effects of temperature (37°C vs 34°C) and dilution (0 or 40% LR or Hextend) on the ability of rFVIIa to improve coagulation in human plasma as measured by thrombin generation and TEG, as well as standard coagulation measures such as PT and aPTT. In general, rFVIIa has been known to induce thrombin generation as part of

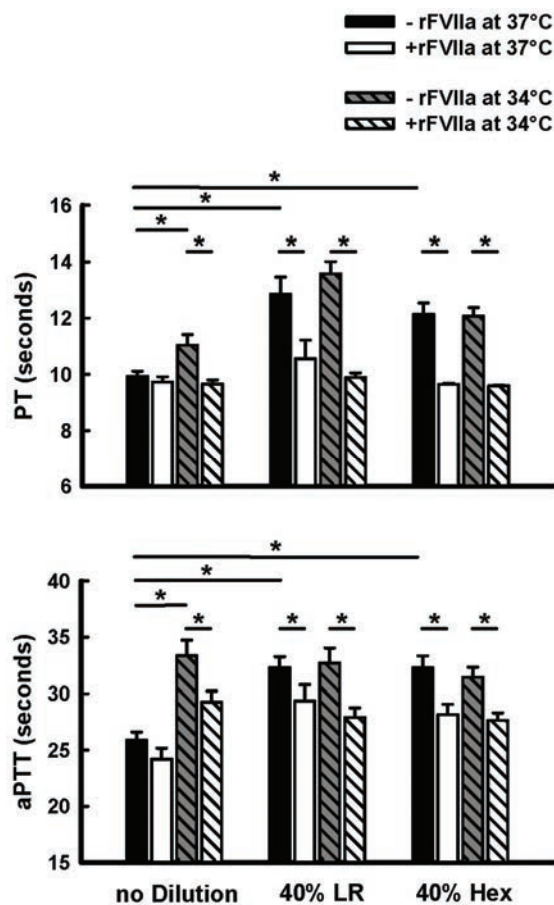


Figure 6. Effect of rFVIIa (1.26µg/ml), temperature (34°C and 37°C), and 40% blood hemodilution (Hextend [Hex] or lactated Ringers [LR]) on PT and aPTT in human plasma in vitro. Values represent Mean±SEM. *= $P<0.05$.

its mechanism of action [29] and combining thrombin generation with TEG provides data on the stability of the fibrin clot, as well [30].

In the present study, temperature, rather than dilution, had the greater effect on thrombin generation. Specifically, the lower incubation temperature of 34°C significantly elevated Peak and ETP as compared to 37°C. Also, the change in Peak thrombin concentration in response to rFVIIa was greater in plasma incubated at the lower temperature. This enhancement of thrombin generation by lower temperatures has been described by others. For example, an elevated ETP at lower temperature was described by Tchaikovski et al., [31] after characterization of a calibrated automated throm-

bography in mouse plasma. An explanation for the effect of temperature was proposed by Hemker et al [32]. They proposed that low temperature affects thrombin inactivation (Thrombin/Antithrombin) more so than thrombin activation (Prothrombin to Thrombin). Because the total amount of thrombin is dependent on the rate it is produced vs. the rate it is bound to antithrombin and inactivated, lowering the incubation temperature would attenuate thrombin inactivation more than thrombin activation, and explain the higher Peak. Several studies, both in vitro and in vivo have reported that rFVIIa can improve coagulation and even reduce bleeding time under hypothermic conditions [33-35].

In contrast to the effects on thrombin generation, dilution with 40% Hextend had the most effect on TEG parameters as compared to 40% lactated Ringer's, or incubation at 34°C. Dilution with 40% Hextend significantly prolonged K-time, and decreased α angle and MA, as compared to control (37°C, 0 Dilution), similar to our previous study [36]. Incubation of whole blood at 34°C had little to no effect on TEG parameters. These data are in agreement with other laboratories showing that 20, 40 and 60% dilution of whole blood with HES 130 significantly reduced α angle and MA [35], and that incubation of whole blood at 32°C had little effect on TEG parameters. Dilution of human blood 40-60% with 5% albumin, isotonic saline, lactated Ringer's or dextran significantly prolong clotting time, and reduced mean clotting firmness as measured by rotational thromboelastometry [37-39]. In the present study, rFVIIa had little effect on PT and aPTT in non-diluted plasma incubated at 37°C. Incubation of plasma at 34°C, or hemodilution, significantly prolonged both PT and aPTT as has been described by others [39, 40]. However, rFVIIa significantly shortened PT in the diluted samples to levels not different from control suggesting that rFVIIa can return PT toward normal levels after dilution of 40%. These observations are consistent with the present observations that rFVIIa had the most pronounced effects on R- or K-time in undiluted or diluted blood at both temperatures. Although rFVIIa had a similar effect on aPTT, it did not return it to control levels. The present results are consistent with other findings that rFVIIa was still effective on TEG parameters or reducing bleeding time in hemodiluted rabbits and pigs [20; 37-43].

In conclusion, we have shown that low temperature had a more significant effect on thrombin generation than did 40% hemodilution. On the other hand, hemodilution, rather than temperature, had a significant effect on TEG. Both temperature and dilution affected PT and aPTT. Furthermore, rFVIIa caused a greater change in thrombin generation at 34°C as compared to 37°C, and a greater change in PT at 40% dilution, suggesting that rFVIIa can be effective at temperatures and levels of hemodilution that may be encountered in a surviving trauma patient.

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Conflict of Interest Statement

None of the authors have any conflicts of interest regarding the contents of this manuscript or the compounds that were examined.

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